

## Quantitative and Qualitative Changes of Phytochemical Composition in Viral-infested *N. tabaccum*

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**Abstract:** Phytochemical studies in relation to development and growth of viral infected *N. tabaccum* cv. White burley are rather restricted. This study analyzed *Tobacco mosaic tobamovirus* affecting plant growth and phytochemical compositions. Quantitative data on branch, leaves formation, stem growth and fresh and dry weights were systematically collected at three months. It was found that the branching rate of TMV infected plant was decline than healthy ones. On the other hand, the plant height, number and area of leaves, fresh weight and dry matter were decreased in TMV infected plants compared to the increase in healthy ones. The TMV infection led to slightly change quantitative and qualitative total chlorophyll, phenolic antioxidant compounds and soluble protein in infected plant. The TMV infection leads to a decrease in chlorophyll a + b, total phenols and soluble protein by rate 47.89, 7.89 and 61.35% in infected leaves. On the contrary TMV infection leads to an increase in carotenes by rate 39.28% in infected tobacco leaves. As well as increase in the phenolic antioxidant compounds in infected leaves. Twenty two phenolic compounds were identified in the leaves of *N. tabaccum* cv. While burley and nineteen in healthy ones by HPLC. N-Hydroxy acyl nornicotine, newly discovered was found in fresh shoot plants with higher amount in healthy compared with TMV infected plants, but not in roots tissues. Nicotine was found in fresh tissues with low concentration in infected root, stem and leaves but was found with high concentration in healthy ones. Nornicotine was found in fresh healthy tissues of roots, stem and leaves but no identified in TMV infected stem and leaves by TLC.

**Key words:** Tobacco plant, TMV, Nicotine HPLC and TLC Chromatography

### INTRODUCTION

Many higher plants accumulate extractable organic substances in quantities sufficient to be economically useful as chemical feed stocks or raw materials for various scientific, technological and commercial applications. Economically important plants serve as sources of industrial oil, resins, tannins, saponins, Nicotine, natural rubber gums, waxes, dyes, pharmaceuticals and many other products<sup>[21]</sup>.

Plant responses to herbivore attack are broadly categorized as direct and indirect defenses and tolerance<sup>[13]</sup>. Nicotine is an alkaloid, which only exists in tobacco plants and accounts for 95% of its total alkaloid content<sup>[10]</sup>. In undamaged tobacco plants, the nicotine concentration is only 0.1-1% its dry mass, while red or simulated herbivore at tobacco leaves may cause an increase up to 1-4%, which is sufficient to deter even nicotine-adapted insects<sup>[20]</sup>. A burley 21 is a genetically stable breeding line of tobacco which is more susceptible to insect damage, probably because of its low nicotine content. Nicotine is synthesized in the

roots and transported in the xylem to the shoot<sup>[2]</sup>. The group of alkaloids contains un acylated nitrogen on the pyrrolidine ring. The major component among them is the [iso-N' (3-hydroxy-12-methyltetradecanayl)]-nornicotine<sup>[16]</sup>. The N-acylnornicotine type at pyridine alkaloids has been found earlier only in air-cured or harvested and aged leaves of *N. tabaccum*<sup>[18,9]</sup>. In the plant kingdom the N-acylated pyrrolidine ring is very rare<sup>[16]</sup>.

However, Baruah and Chowfla,<sup>[3]</sup> and Ali *et al*,<sup>[1]</sup> suggest that there are high contents of polyphenols present in healthy plants. On the contrary, Suresk *et al*<sup>[26]</sup> state that there are higher amounts of total polyphenols in virus infected plants. By way of oxidation of indole-3-acetic acid up regulated peroxidases might also be responsible for growth reduction and malformations in virus infected plants<sup>[24]</sup>. We initiated a study on the distribution and synthesis of the alkaloid in the plant, and the effect of viral infection on alkaloid and nicotine content, the most important group of secondary metabolites on the selected plant *N. tabaccum* cv. White burley.

## MATERIAL AND METHODS

**Plants:** The seeds of *N. tabaccum* cv. White burley were kindly provided by Virology Lab., Fac. Agric., Ain Shams Univ., the seeds germinated in a mixture of 60% peat culture and 20% perlite and grown in a seed bed under greenhouse for 60 days. Before transfer tobacco seedlings into pots, the seedlings were washed with tap water until all substrate was removed from the roots. The tobacco plants were grown in earthenware pots (30 cm in diameter), 3 plants/pot using standard soil mixture and were fertilized (20-20-20 NPK) once per 2 weeks.

**Virus:** *Tobacco mosaic tobamovirus* (TMV) was kindly provided by the Virology Lab., Microbiology Dept., Fac. Agric., Ain Shams Univ. Twenty five tobacco plants were mechanically inoculated with infectious sap and kept under greenhouse conditions. During the experimental period, the average temperature was 28-30°C. Light day and 18-22°C at night day. The plants were watered daily, initially with full strength nutrient solution.

**Conformation of TMV:** Infectious sap from TMV infected tobacco plants was extract in 0.05 M phosphate buffer pH 7.2 was used for mechanical inoculation of differential hosts *N. tabaccum* cv. White burley, *N. glutinosa*, *Lycopersicon esculentum* cv. Castle rock, *Datura stramonium*, and *Chenopodium amaranticolor* and *Gomphrena globosa*. The inoculated plants were kept in greenhouse at 28-30°C during the day and 18-20°C at night. Inoculated *N. tabaccum* cv. White burley and different hosts were tested against TMV by indirect ELISA according to Koenig<sup>[14]</sup>. TMV specific polyclonal inhibitors used in this study was obtained from plant Virology Lab. Agric. Dept., Fac. Agric., Ain Shams Univ.

Morphological characteristics and active constituent of plants were determined after 3 months of cultivation. The recorded morphological parameters were plant height, number of leaves plant<sup>-1</sup>, fresh weight and dry matter.

**Biochemical Analysis:** Chlorophyll a, b, total chlorophyll and carotenes were estimated according to method mentioned by Jayaraman<sup>[12]</sup>. Total polyphenols were assayed as mentioned by Malik and Singh<sup>[17]</sup>. The nicotine concentration was lysed by the ultraviolet absorption method<sup>[27]</sup>. Protein determination was accomplished according to Bradford<sup>[4]</sup> using bovine serum albumin as standard.

**Extraction of Nicotine Alkaloids:** Ten grams of healthy or infected plant leaves, stems, or roots were

cut into small pieces and ground in a mortar with sand in 50 ml chloroform containing 1 ml NH<sub>2</sub>OH. The homogenized material was kept over night at room temperature and then filtered through cloth and partitioned against 3 x 30 ml water acidified with HCl to pH 2. The chloroform fraction was evaporated under vacuum and when the leaves had not been previously washed with acetonitrile. The fraction was the source of N-hydroxyacetylornicotine. The acetic water was collected, the pH adjusted to 12 with NaOH and partitioned against chloroform. The latter was then evaporated (base fraction). This fraction was the source of the nicotine and the nornicotine.

**TLC Chromatography:** TLC plates (Kieselge 60) were developed in chloroform-methanol (100; 40) and chloroform-methanol (NH<sub>4</sub>OH 90:10:1) solvents, sprayed with p-aminobenzoic acid and put into cyanogen bromide vapor. The alkaloids were identified by R<sub>f</sub> value and color with the help of standards (nicotine) Sigma). The internal nicotine and nornicotine content of homogenized leaves was chloroform extracted. The chloroform extracted. The alkaloid residue TLC purified eluted, dried and dissolved in 100 µl ethanol. The quantity was estimated by visual comparison of the thin layer chromatograms of dilutions of standards. Aliquot parts of the obtained alkaloid concentrates were either diluted up to 1 ml with 0.1 N HCl and measured by A at 260 nm.

**HPLC Analysis:** Phenolic extracts intended for the HPLC analysis were prepared according to the method applied by Candela *et al.*<sup>[5]</sup>. Phenolic compounds analysis was conducted with HPLC apparatus Hewlett. Packard HP (Molde 1100) liquid chromatograph, with a reverse-phase hypersil C-18 (5 µm packing, 46 x 150 mm) column. The HPLC connected to UV/V/S detector. HPLC grade solvent used as follow. Solvent A, 0.5% acetic acid in bidistilled water at pH 2.8, solvent B, 0.5% acetic acid in 99.5% acetonitrile. Gradient development was linear starting with A and ending with B during 50 min, at flow rate 0.3 ml/min, with detector wave length 254 nm, and injection volume 5 µl, in the Lab of General Organization for Agric. Equalization Fund (G.O.A.E.F).

Statistical analysis was carried out using student's t-test (P < 0.05).

## RESULTS AND DISCUSSION

### Results:

**TMV Isolate Confirmation:**Data in Table (1) showed that TMV isolate gave systemic symptoms on *N. tabaccum* cv. White burley (Fig. 1) in the form vein banding and mild mosaic followed by seven mosaic,

**Table 1:** Response of differential hosts inoculated with TMV isolate.

Plant species	Host response	Indirect ELISA* (at 405 nm)
<i>Ch. Amaranticolor</i>	NLL	1.450
<i>D. stramonium</i>	NLL	1.525
<i>G. globosa</i>	ChLL	0.957
<i>L. esculentum</i> cv. Castle rock	mM, LC	0.975
<i>N. tabaccum</i> cv. White burley	VB,SM,RL,D.	1.250
<i>N. glutinosa</i>	NLL	0.850

CHLL= Chlorotic local lesion,

D = Dwarfing; mM = mild mosaic

NLL = Necrotic local lesion; RL= Reduction leaf blade;

SM = Severe mosaic;

VB = Vein banding.

\* = Negative control (healthy) 0.325



**Fig. 1:** *N. tabaccum* cv. White burley inoculated with TMV appeared severe mosaic, malformation, reduction of leave size and dwarfing (b) compared with healthy plants (a).

malformation (reduction of leaf blade) and dwarfing. *L. esculantum* cv. Castle rock showed mild mosaic and leaf carl after 15-20 day post-inoculation. Necrotic local lesions were appeared on *D. stramonium*, *Ch. Amaranticolor* and *N. glutinosa* as well chlorotic local lesions were appeared on *G. globosa*.

The virus infection was confirmed used indirect ELISA. Results in Table (1) show positive values for TMV infected tobacco plants cv. White burley, while healthy plants gave negative results. In addition differential hosts, *L. esculantum*, *N. glutinosa*, *D. stramonium*, *Ch. Amaranticolor* and *G. globosa* gave positive ELISA results.

**TMV Isolate Propagation:** The virus isolate was propagated in *N. tabaccum* cv. White burley by mechanical inoculation. Data showed severe mosaic, blister, vein banding, malformation and dwarfing.

These result were confirmed by ELISA whereas gave positive values against polyclonal antibodies specific TMV.

**The Influence of TMV Infection on the Growth of Tobacco Plants:** The data in Table (2) illustrate the morphological difference between healthy and TMV infected *N. tabaccum* cv. White burley. It was found that the highest rate of branching was obtained in healthy plant being 6 branches plant<sup>-1</sup>, while infected plant gave 4 branches plant<sup>-1</sup>. The number of leaves per plant was influenced by TMV infection where 25 leaves plant<sup>-1</sup> were obtained in infected plant while in the case of healthy ones it reached 20 leaves plant<sup>-1</sup> after 3 months of cultivation. The decrease in plant height 42 cm was obtained in infected plant comparison with healthy plant as 60 cm. TMV infection of *N. tobaccum* cv. White burley due to in a

**Table 2:** Effect of TMV infection on morphological characters of *N. tabaccum* cv. White burley plants.

Plant species	Healthy plant	Infected plant	% of reduction
- Rate of branching plant <sup>-1</sup>	600	4.00	33.33
- No. of leaves plant <sup>-1</sup>	25.00	20.0	20.00
- Leaf area (cm)	6.50	3.90	40.00
Plant height (cm)	60.00	42.00	30.00
Fresh weight plant <sup>-1</sup> (g)	75.00	48.60	35.20
Dry matter plant <sup>-1</sup> (g)	9.50	3.24	65.89

decrease of 40% in leaf area. The fresh and dry weight of *N. tabaccum* plant was negatively influenced by TMV infection. The fresh weight and dry matter were decreased by 35.20 and 65.89% respectively.

**Phytochemical Compositions:** Data in Table (3) reveal the biochemical changes in TMV infected *N. tabaccum* cv. White burley comparison with healthy ones. It was found that chlorophyll a, b and a + b were decreased by rate 47.39; 48.50 and 47.89 respectively. While carotenes content were increased in TMV inoculated plants by rate 39.28% related to healthy ones. Despite healthy plant has larger amount of chlorophyll a than b but chlorophyll a:b ratio was decreased to be equalized after inoculation. Moreover, the color becomes orange of carotene and yellow green of chlorophyll b. The leaves corresponding to healthy ones. The total polyphenol content is slightly decreased in inoculated plants by rate 7.89% compared with healthy ones, (Table 3). Leaves soluble protein was decreased to be 61.35 in inoculated plants compared with healthy ones (Table 3).

**HPLC Analysis of Phenolic Compounds:** The phenolic and oxidant compounds is slightly change quantitative and qualitative in TMV infected tabacum plants related to healthy ones. HPLC analysis revealed the presence of about 19 phenol compounds in healthy of *N. tabaccum* leaves and 22 compounds in infected ones compared to standard phenol compounds (Fig. 2). The fraction and lyses of phenol compounds in healthy and infected leaves was presented in Table (4) and Fig. (3). The virus infection due to increase amount of Garlic acid; P-hydroxy benzole acid; phenol, salicylic acid cinnamic acid and Kaemphenol compared to healthy leaves.

Alkaloids from healthy and TMV infected *N. tabaccum* cv. White burley have been extracts from leaves, stem and roots chloroform, NH<sub>4</sub>OH and acetonitrile. We observed nicotine, nornicotine and N-hydroxyacrylnornicotine (Fig. 4). The fresh tissues of roots did not contain N-hydroxyacrylnornicotine of infected or healthy plants, while it contained nicotine and nornicotine. The leaves and stems contained this

alkaloid with high amount in healthy plant (Table 5, and Fig. 3, Lane d, f) than infected ones (Table 5, Fig. 3 Lane c and e) as did the leaves and stems. The amount of this substance was about half of that nornicotine. There was more acylated nornicotine at the leaves and stem. The tissues of leaves, stems and roots contains nicotine, nornicotine and some unidentified alkaloids one of which is apparently substance (by R<sub>f</sub> and color). The order of alkaloid content per fresh weight was high in the root than leaves and stems respectively. About 90% of the total alkaloid content was nicotine and nornicotine. The nornicotine concentration was about 15% of nicotinic in the leaves higher in the root but lower is the stem, which is consist of with other reports. None of these parts of plant organs contain N-hydroxyacrylnornicotine (Figs. 3b, d, f).

**Discussion:** The virus TMV isolate was confirmed, differential hosts and indirect ELISA were used, data in Table (1) show different infections local infection on *Ch. Amaranticolor*, *D. stramonium*, *G. globosa*; *N. glutinosa* and systemic infection on *L. esculantum* cv. Castle rock and *N. tabaccum* cv. White burley, as well as positive values with indirect ELISA against polyclonal antibodies specific TMV. Several investigators showed that, differential hosts and ELISA the most easily and widely for TMV detection and conformation<sup>[28,7]</sup>.

Data obtained on Table (2 and Fig 1), illustrate presence of mild mosaic (chlorophyll reduction) appeared after 10 days followed by severe mosaic, malformation and reduction of leaf area after 20 days of TMV inoculation on *N. tabaccum* cv. White burley. Phytochemical analysis reveals lowering in chlorophyll a, b and a + b. Chlorophyll lowering was noticed more in a than b and the chlorophyll ratio a:b was decreased in infected leaves. Although in healthy plant chlorophyll a was more than b in general, but this ratio was decreased to be equal after infection, which mean that virus infection led to destroy chlorophyll a at the expense of b. The decrease in chlorophyll a/b ratio is considered to be a symptom of oxidative stress condition this decrease in the ratio after virus infection

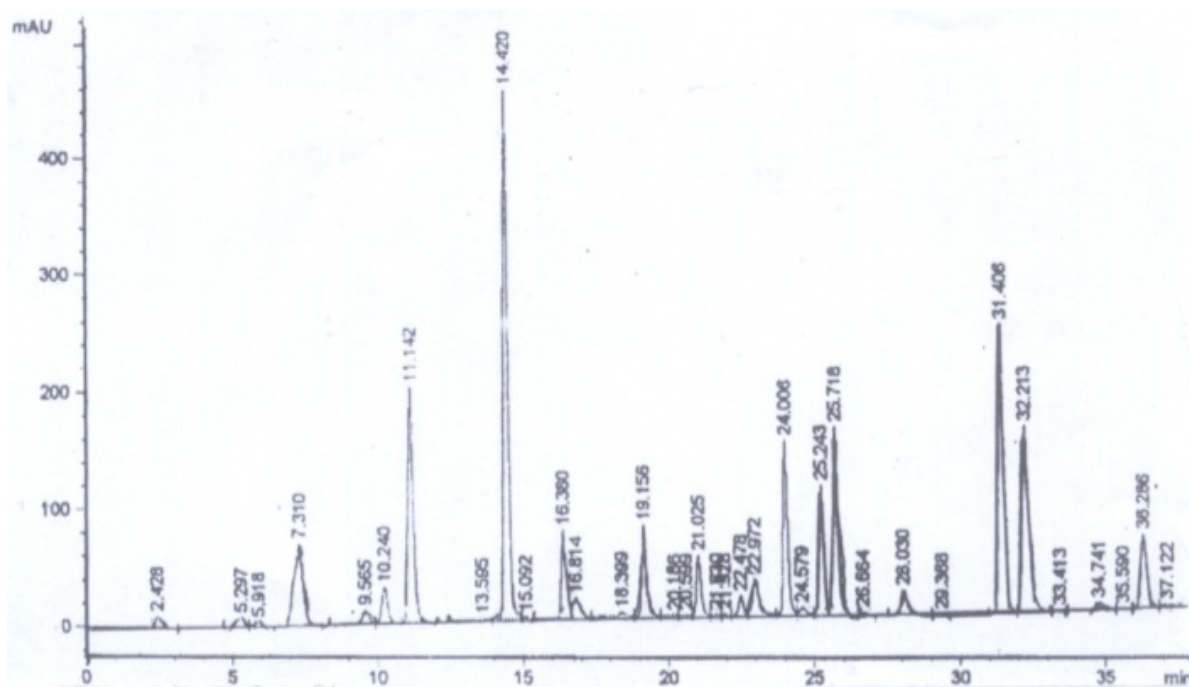
**Table 3:** Phytochemical changes in *N. tabaccum* cv. White burley plants inoculated with TMV.

Phytochemical	Healthy leaves	Infected leaves	% of changes
Chlorophyll a*	80.50±2.5	42.35±1.75	-47.39
Chlorophyll b*	65.25±1.85	33.60±1.36	-48.50
Chlorophyll a+b*	145.75±35.2	75.55±2.72	-47.89
Carotenes*	4.25±0.50	7.00±0.85	+39.28
Total phenols**	9.25±1.25	8.52±0.90	-7.89
Soluble protein**	40.75±2.50	15.75±1.85	-61.35

\* mg/100 g fresh weight

\*\* mg/g fresh weight

The results were expressed at mean ± standard error.



**Fig. 2:** Chromatogram of standard phenolic compounds achieved by reverse-phase HPLC, and its retention time (Rt).

might be due to the generation of reactive oxygen species (ROS) causing damage to chlorophyll a. That is mean the plant failed to capture the light and so photosynthesis will decrease or stopped. The decreasing in chlorophyll and the increasing in carotenes interpret the color change after infection.

Data in Table (2) and Fig. (1) illustrate the morphological difference between healthy and infected *N. tabaccum* with TMV. It was found that, the highest rate of plant growth was obtained in healthy plant compared with TMV-infected plants. As well as the phytochemical changes, TMV infected plant. Comparison with healthy, the total polyphenol and protein content is decreased in inoculated plants with TMV compared with healthy plants.

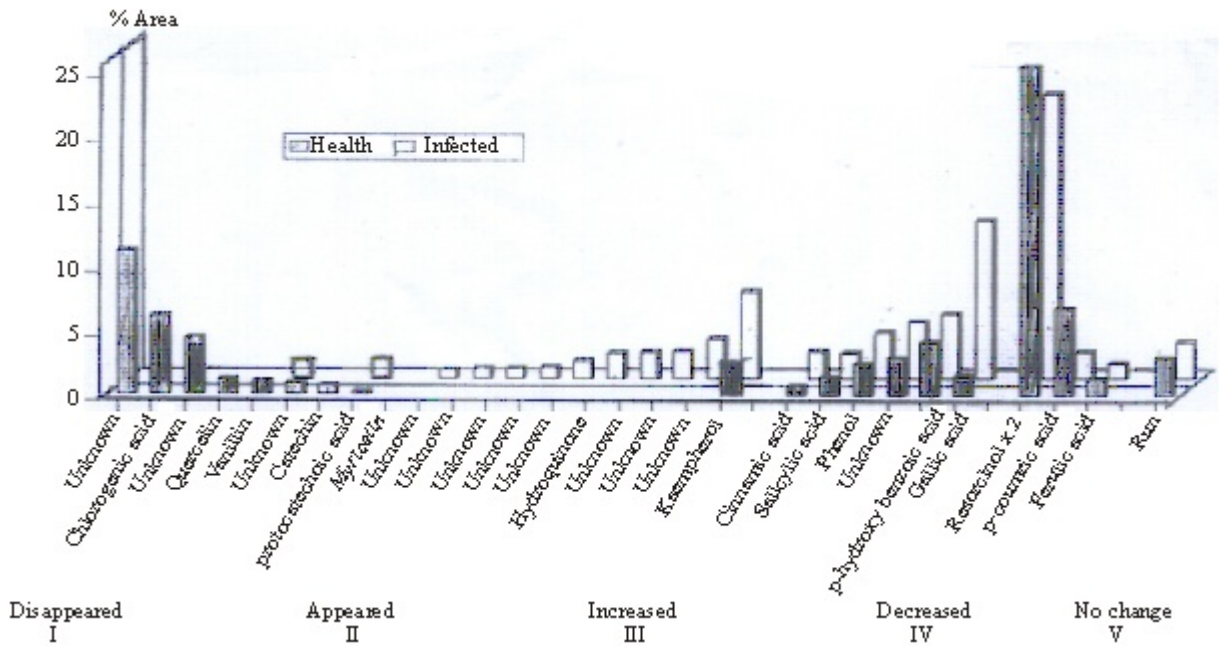
All the various morphological and physiological of TMV infection are induced indirectly by the virus. Many workers described differences in comparison or in rate of some process between healthy and virus infected tissues. Effects community found was: (I) a decrease in rate of photosynthesis often associated with a decrease in protosynthetic pigments, chloroplasts, ribosomes and fraction 1 protein, (II) an increase in respiratory rate (III) an increase in the activity of certain enzymes, particularly polyoxidase and the accumulation of oxidized polyphenols and (IV) a decreased or increased activity of plant growth regulators. Many of the changes in host plant metabolism noted above are probably secondary consequences of virus infection, not essential for virus

**Table 4:** Phenol compounds analysis (% area) in healthy and TMV infected *N. tabaccum* cv. White burley.

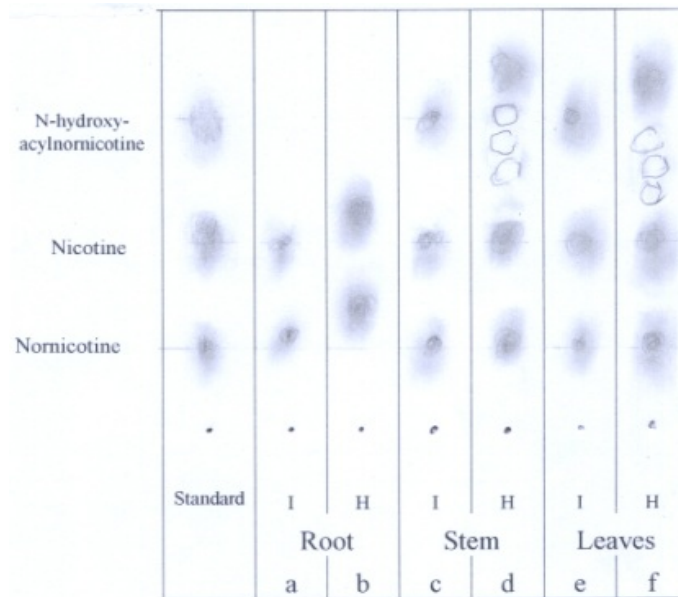
Retention time	Phenolic compounds	Healthy leaves	Infected leaves
3.25	Unknown	-	0.75
5.30	Pyrogalic acid	-	-
5.90	Hydroquinone	-	1.93
7.30	Galic acid	1.48	12.30
9.55	Resorcinol	25.85	23.10
11.15	Protocatechuic acid	0.22	-
14.40	P-hydroxy benzoic acid	4.75	5.25
15.10	Unknown	2.81	4.38
15.50	Unknown	10.75	2.50
16.38	Chlorogenic acid	6.08	-
16.81	Catechin	0.71	-
18.40	Phenol	1.40	2.75
19.15	Vanilin	4.02	-
21.02	P-Coumaric acid	6.72	2.00
21.65	Unknown	-	0.77
22.50	Ferulic acid	1.24	1.57
22.95	Salicylic acid	1.40	2.05
24.00	Rutin	2.78	2.78
25.25	O-Coumaric acid	-	-
25.70	Coumarin	-	-
28.05	Myricetin	-	0.90
31.40	Cinnamic acid	0.68	2.09
32.20	Quercetin	1.18	-
34.75	Unknown	-	3.06
34.99	Unknown	-	1.33
36.30	Kaemphenol	2.50	4.75
37.10	Unknown	-	2.08
38.15	Unknown	-	0.87
39.25	Unknown	-	2.02
39.70	Unknown	-	0.66
40.29	Unknown	4.38	-
40.50	Unknown	0.86	-

**Table 5:** Alkaloids production by root, stem and leaves of *N. tabacum* healthy and infected with TMV.

Plant tissue	N-hydroxyacetylornicotine ( $\mu\text{m/l}$ )		Nicotine ( $\mu\text{m/l}$ )		Nornicotine ( $\mu\text{m/l}$ )	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
	Leaves	375	150	775	425	710
Stems	425	315	795	350	90	95
Roots	-	-	672	325	85	75



**Fig. 3:** Phenolic *N. tabacum* cv. White burley analyzed by HPLC in healthy and infected leaves of inoculated with TMV.



**Fig. 4:** Diagram illustrate the nicotine alkaloid in fresh root, stem, and leaves from healthy (H) and TMV inoculated (I) *N. tabacum* with acetone for 5 min. standard N-hydroxyacetylornicotine, nicotine and nornicotine

replication. In many virus diseases, the general pattern of metabolic change appears to resemble an accelerated aging process. There are many variables to be taken into account when using intact plant organs. More viruses are usually produced in them and they are most often used for exponential work, i.e. A mosaic virus infection of *Solanum khasianum*, Clarke reduced the fruit content of solanone (a medically useful alkaloid) to about one half<sup>[22]</sup>, *Potato virus Y* infected *Datura metel*<sup>[8]</sup> and TMV infected *N. glutinosa*<sup>[1]</sup>.

Viruses are economically important only when they cause some significant deviation from normal growth of the plant, reduction in plant height, size of leaves, flowers, roots and a shortening of petioles and internodes reduced by virus infection. A reduction in total medically useful alkaloids of leaves is a common feature and an important economic aspect of virus disease. The decrease in alkaloid contents may be due to reaction in plant total fresh weight specially leaves<sup>[8]</sup>.

Also it was found that virus infection led to induce protein synthesis resistance to pathogen in tobacco plants can be induced by salicylic acid. It has been claimed that salicylic acid act as the internal signal that triggers general resistance in plants and induces the expression of messenger RNA, which presumably direct the synthesis of the PR proteins<sup>[19]</sup> showed an increase in endogenous salicylic acid level in tobacco mosaic virus which caused a hypersensitive response with systemic induction of PR proteins.

Total polyphenols content in increased in healthy compared to infected leaves, in addition, the total polyphenols and types were changed. Consequently, it could be concluded that viral infections do not affect only on the synthesis of polyphenols but also have effects on the distribution type of polyphenols in an infected leaves. Some phenolic compounds do not increase but others show increase and these might be responsible for the resistance in infected plant. In the present study, after infection with virus, phenolic compounds could be classified into five main groups, 1<sup>st</sup> phenolic which disappeared, 2<sup>nd</sup> appeared, 3<sup>rd</sup> increased, 4<sup>th</sup> decreased, 5<sup>th</sup> not changed (Fig. 3).

The localization and the synthesis of N-hydroxy acrylornicoline is described here in one of the species of section Repondae. This substance, only recently described from fresh plant tissue<sup>[11]</sup>, has been completely missed in many surveys<sup>[25]</sup>. The failure of the alkaloid to be detected in fresh tissue previously may be because this chemical is hardly soluble in water and cannot be recovered from the so-called base fraction as can the other nicotine alkaloids.

Nicotine which is the main alkaloid in the interval tissue is produced by the aerial part of the plant where it is demethylated to nornicotine<sup>[6]</sup>. This synthesis route was also found in *N. tabaccum* cv. White burley in the

present study. The plausibility of nornicotine as the direct precursor to N-hydroxy acyl nornicotine is strengthened when it is noted that the specific activity of administered nornicotine was 16.000 times less than that of the administered nicotine, yet the specific activity of the B-hydroxy acyl nornicotine obtained from nornicotine was higher than for nicotine. The amounts of endogenous nicotine and nornicotine in these leaves prior to feeding was in the same order of magnitude.

Exudate on the leaf surface may not contain enzymes for alkaloid degradation, since no alkaloid breakdown products soluble in aceto nitrite were detected in the exudates. Thus, the N-hydroxy acyl nicotine can build up in the exudates to a very high concentration for presentation to herbivorous viruses without degradation or toxicity to the plant.

In the leaf wounding experiment with different numbers of leaf punctures a significant and positive relationship between the number of leaf puncture and nicotine accumulation was found<sup>[22]</sup>. In the present study, however, virus infection caused only a slight decrease in nicotine concentration. This slight decrease in nicotine was sources of auxin in the plant<sup>[26]</sup>.

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